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DNA Clones: Development and Application to Breast Cancer Genomes

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13. ABSTRACT (Maximum 200) The amplification of chromosome 20q occurs in 15-20% of primary breast tumors and correlates with poor prognosis indicating that this region may contains gene(s) involved in breast cancer development. In order to define this region with high precision, we are developing methods to perform comparative genomic hybridization to dense clone arrays. Tumor and reference DNAs are labeled with different fluorochromes and simultaneously hybridized to an immobilized set of mapped clones spanning the length of chromosome 20. Copy number abnormalities involving a clone are indicated by changes in the tumor/normal fluorescence ratio compared to average. By titrating λ DNA probe in a background of human genomic DNA, we show that this method possesses sufficient sensitivity to detect single copy deletions as well as amplification, and is linear over several orders of magnitude. Using manually prepared 30-60 clones arrays, breast cancer cell lines and primary tumors reveal that 20q is suprisingly complex, possessing at least five regions of amplification and possibly one deletion. Finally, an automated printer is being developed to prepare large numbers (~100) of arrays in parallel of higher (up to 10^4 spots/cm ²) density.					
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FOREWORD

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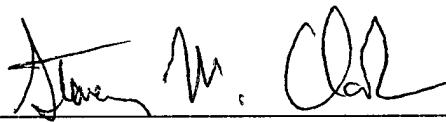
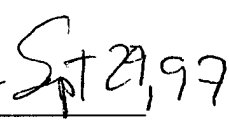
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Introduction

The amplification or deletion of defined chromosomal regions is a common and important event in cancer progression, contributing to tumor malignancy, progression and drug resistance (1). Several studies indicate that the amplification of portions of the long arm of chromosome 20 may be important in breast and other cancers. Comparative genomic hybridization (CGH) shows that 20q13 is amplified in 12-18% of primary breast tumors (2, 3) and high level amplification correlates with high histological tumor grade, DNA aneuploidy, high S-phase fraction and poor prognosis in breast cancers (4). *In vitro* immortalization experiments using HPV16 E7 transformed human primary cells show that low level 20q gains are associated with overcoming the cellular senescence block and high level 20q13.2 amplification correlates with genomic instability (5). Previously known oncogenes at 20q13 were excluded using fluorescence *in situ* hybridization (FISH) (6), suggesting that this region may harbor a novel oncogene with functional importance in breast cancer progression. However, the low (1-10 Mbp) resolution and/or the labor intensive aspect of these methods make the definition the critical gene(s) involved difficult.

Under this postdoctoral fellowship, I am developing a CGH-based method capable of rapidly scanning tumor genomes for copy number abnormalities (CNAs) with gene-by-gene (<100 kbp) resolution and am applying this method to the 20q region in breast cancer. In this method, tumor and normal DNAs are labeled with different fluorochromes and simultaneously hybridized to arrays of clones spanning the region of interest (i.e. all of chromosome 20 at low resolution or the 20q13 region at high resolution). CNAs are inferred by changes in the fluorescence ratio of a given clone compared to the average. Initial studies of 5 breast tumors

and cell line BT474 using ~30 clone arrays confirm that 20q contains several independent regions of amplification. The CNAs observed in cancer are complex and possess a partially random nature (7). Thus, many tumors must be evaluated with high (<100kbp) resolution to define the statistically significant region(s). For these reasons, I am developing an automated printer to manufacture large numbers of arrays of higher clone density.

Experimental Methods

Slide Preparation. Glass or fused silica slides are soaked for one day in HCl:MeOH = 1:1 (v/v), soaked for an additional day in sulfuric acid, washed with water, reacted with 3-aminopropyltrimethoxysilane (0.1% (v/v) in acetone for 2 min.), washed with acetone, and cured at 100C for 15 minutes. Slides may be recycled after washing with fresh 'piranha' ($\text{H}_2\text{O}_2:\text{H}_2\text{SO}_4=1:1$ (v/v)).

Target Preparation. Ten μg of cosmid, P1, or BAC DNA is precipitated and resuspended in 5 μl of the target solvent ($\text{H}_2\text{O}:\text{DMSO}:\text{Nitrocellulose} = 1:4:0.0033\%$ (v/v/w)). These targets are either placed in a microcentrifuge tube for manual array preparation or in a 864-microwell polycarbonate plate (Vorhies Technologies) for printing using the capillary printing head being developed in this project (see below).

Target Printing. Target spots are printed manually using a pulled glass capillary or using the printing head shown in Fig 4. 3 x 3 spring-loaded fused silica capillaries (Polymicro Technologies, 365 μm o.d. 75 μm i.d.) are positioned 3 mm. apart within the printing head to match the spacing of the 864-well plate. The capillary tips are sharpened to ~125 μm o.d. using the homemade miniature lathe shown in Fig. 5. These capillaries

produce ~150 diameter spots, and we have similarly polished 25 μm i.d. capillaries to ~50 μm o.d., producing 50 μm diameter spots. The top of the capillaries are linked to a manifold by press-fit flexible tubing.

To print, the head is lowered for several seconds into the microwell plate and clone solutions are drawn into the capillaries by capillary action. This loads enough target solution to print ~60 spots. The capillaries are withdrawn and the manifold pressure is increased and held to a slight positive pressure (~1.03 atm) to effect printing. This back pressure is produced by compressing a hand pipettor by a fixed volume. Rubber cement is then placed around the perimeter of the spots to confine the hybridization solution.

Array Hybridization. Tumor and normal genomic DNA are labeled by nick translation using dCTP-FITC and dCTP-Texas Red, respectively. The probe size is determined on an agarose gel and the nick translation reaction is continued if the probe is longer than ~600 bp. After purification on a Sephadex G-50 column, 200 ng of each of these probes is mixed with 35 μg of C_{ot} -1 blocking DNA and precipitated. The pellet is resuspended in 10 μl of hybridization mix (10 μg / μl yeast tRNA, 4% (w/v) SDS, 10% (w/v) sodium dextran sulfate, 50% formamide, 2X SSC pH 7.0). This solution is denatured at 75C for 10 minutes and incubated at 37C for 1 hour to block repetitive sequences. This mixture is then placed on the array and incubated with rocking for one day at 37C. After hybridization, the slides are washed with 50% formamide, 2XSSC at 45C for 30 min. washed with PN solution, the rubber cement is removed, the slides rinsed with water and dried at 37C. The array is then counterstained with DAPI and sealed with a coverslip.

Array Quantification. We have developed a 1X macroscope (8, 9) to rapidly collect data from the relatively large array areas (10 X 10 mm). Analysis software finds the target perimeters using the DAPI signal, numbers the targets, performs background corrections and outputs the test/reference ratios. The quality of data from an individual spot is determined by the correlation between the test and reference signals within the spot and spots with low correlation are rejected.

Results and Discussion

Proposal Experimental Plan 1: Optimization of target attachment chemistry. The ideal attachment chemistry will produce targets that 1.) are stable throughout the course of hybridization, 2.) produce no background fluorescence or nonspecific binding of probe, 3.) can be prepared from short (<500 bp) fragments such as PCR products, and 4.) possess sufficient stability such that the bound probe can be melted off and the array can be reused.

Currently, our group forms targets by dissolving the DNA in a DMSO solution containing a small amount of nitrocellulose and spotting this solution onto aminosilyated slides. The nitrocellulose has been shown to stabilize the DNA targets. This chemistry satisfies criteria 1.) and 2.) above. Avenues included in the Proposal and other approaches were attempted to try to satisfy the other criteria. Chemistries to increase the binding affinity of target DNA to the slide were compared. Advidin-biotin coupling was abandoned due to increased nonspecific background fluorescence. Surprisingly, I found that primary and quaternary aminosilyation, poly-L-lysine binding, and the polymerization of primary and quaternary acrylamino groups from surface vinyl groups, yielded similar target stability by DAPI staining. Evaluation of the target DNA concentration and

the target drop size shows that the spots formed are composed of many layers of DNA (~ 20 bp/nm²). The above chemistries are only modifications to the surface of the slide and are thus only capable of affecting the interactions with the lowest layer or so of DNA. The bulk of the target DNA, however is not in contact with the surface and is thus insensitive to these chemistries. Indeed, moderate, albeit somewhat decreased, target stability was observed using bare glass slides.

These experiments suggested that the way to increase the stability of the DNA targets is immobilize the entire target volume. The current chemistry may be effective because the targets contain a 3D network of DNA stabilized by nitrocellulose. A variety of attempts to create a covalent (and supposedly more stable) DNA-copolymer network were attempted using acrylamides as the stabilizing copolymer. Attempts were made to attach acryl groups to denatured DNA using the activated monomers methacryloylchloride, methacrolein and glycidyl methacrylate. The DNA was precipitated and suspended in a solution of acrylamide and APS/TEMED initiator in an attempt to see if the DNA was crosslinked in a insoluble form. Gel analysis showed that this did not occur; this failure was most likely due to the poor yield of the DNA activation step. The addition of poly-L-lysine to the target solution resulted in rapid precipitation of the target DNA, as expected. However, when the targets are first dried onto the slide and then washed with a dilute poly-L-lysine solution in 70% ethanol, the targets are stabilized such that DNA less than 500 bp is bound as effectively as larger DNA (criterion 3). However, hybridization experiments showed that this treatment resulted in a small amount of nonspecific probe binding. The ease and reproducibility of the poly-L-lysine/ethanol procedure is a significant step forward and ideas to remove

this nonspecific binding will be discussed in the 'Recommendations in Relation to the Statement of Work' section below.

Proposal Experimental Plan 2: Hybridization performance using genomes with defined copy number changes: Method validation.

A series of model experiments were performed to quantify the performance of our hybridizations. Using λ as a test sequence in a background of human genomic DNA, we showed that this method possesses sufficient sensitivity to detect single copy deletions as well as the linearity to detect amplifications over several orders of magnitude (Figure 1). The performance of full genome probes was tested by manually spotting ~25 clones spanning the length of chromosome 20 as shown in figure 2A. The clones are spaced at ~1-3 Mbp intervals except at 20q13.2, where four clones from a 1 Mbp contig were included. Figure 2B displays data from control hybridizations using normal test DNA showing the expected flat ratios along the chromosome. Data from the breast cancer cell line BT474 are shown in Figure 2C and are in close agreement with prior FISH results on this chromosome (data not shown). Further method validation with the other cell lines ((45,X0)-(45,XXXXX), 600MPE) described in the Proposal was not undertaken. These experiments demonstrate that this method possesses sufficient sensitivity and linearity and produces data in excellent agreement with established protocols.

The Proposal Experimental Plan speculated that cationic detergents such as cetyltrimethylammonium bromide may decrease the time of hybridization time by orders of magnitude as previously shown by others (10). However, I found that the addition of this reagent resulted in the precipitation of DNA in the hybridization solution over a wide range in

concentration. This is most likely due to the high nucleic acid concentrations ($\sim 10 \mu\text{g}/\mu\text{l}$) we use.

Proposal Experimental Plan 3: Application to primary breast tumors and cell lines. A small set of breast tumors and cell lines were selected for initial study on the basis of previously described 20q13 amplification using FISH. These samples were used in an attempt to define the minimal region of amplification on chromosome 20 with coarse ($\sim 1\text{-}3 \text{ Mbp}$) resolution. Figure 2C shows that cell line BT474 displays a complex pattern of amplification, with five independent regions of amplification (regions A-E) and possibly one deleted region (region F). The tumor S50 is amplified in a manner similar to BT474 and tumors S6, S21, S59, S234 posses different subsets of these regions (Figure 2D). These results are in concordance with previous FISH results which define three independently amplified regions (regions A, B and C) (11). These results highlight the power of CGH to dense arrays, showing that a single array hybridization can produce as much data as a large number (>20) of independent FISH hybridizations.

Development of the array printer. We are developing an automated array printer in order to scan more tumors with higher resolution. A benchtop prototype (Fig. 3) was used to develop the printing head that will later attached to a robotic system.

A significant amount of work went into developing the printing pins. Fused silica capillaries could load the target solution, but were unable to dispense when touched to the slide. Stainless steel tubes could dispense, but only erratically. I then tried methods to force the pin tips to be wet with target solution. Initially, a passive method was attempted, whereby the interior of the capillary was coated with a hydrophobic film (i.e.

dimethyldichlorosilane or octadecyltrichlorosilane) and the tip was left hydrophillic (bare silica) so the resulting chemical pressure would wet the tip with (hydrophillic) target solution. This method worked in principle but was abandoned because of poor stability of the interior films in the DMSO target solution. In contrast, I found that printing with constant back pressure on the pins produces spots of uniform size in a highly reliable fashion. Spots are created by placing the fused silica capillaries in the target solution for several seconds under ambient pressure, removing the tips from the target solution, increasing the back pressure slightly and briefly touching the capillary tips to the glass slide. Holding the back pressure to a constant value permits the printing of ~60 consecutive spots. After printing each set of clones, the capillaries are vacuum washed with water and acetone. Currently, the head consists of nine spring loaded capillaries (Fig. 4), so clones are printed in sets of nine from the 864-well microwell plate.

Arrays consisting of identical λ spots were prepared to quantify the performance of the printing and imaging processes. Our group has developed a 1X microscope (9, 12) to quickly image the large (1 cm X 1 cm) arrays. Figure 7 shows the fluorescence images from a 225 spot test array. Here, the spots are printed with 500 μ m spacing and are of uniform size (STD of spot area = 15% by DAPI). However, it is apparent that there are some problems with the flatness of the optical field in the FITC and Texas Red channels. This array was used as an optical standard to correct these problems such that the standard deviation in fluorescence ratios is currently less than 10% across the array.

The diameter of the targets produced is roughly the size of the area of the capillary tip. In order to make smaller spots, I built a mini-lathe

(Fig. 5) to polish the capillary tips to small outside diameters (Fig. 6). λ arrays were printed with 25 μm i.d. tips on 100 μm spacing (Fig. 8). The capillary back pressure must be increased when smaller i.d. capillaries are used and scales as the i.d.², as expected from hydrodynamics. Using this model system, I have found no change in the intensity of bound probe as a function of spot size. These experiments show that the preparation of higher density (10,000 or more spots/cm²) arrays presents no major technical difficulties.

Recommendations in Relation to the Statement of Work

For the second and final year of my postdoctoral work I will pursue the areas necessary to define the minimal region(s) of CNA on chromosome 20 in breast cancer. This will involve further development of technologies to perform CGH onto dense arrays as described below:

Improving the target attachment chemistry. As stated above, the current target attachment is sufficient for our current clones, yet not ideal, for short DNAs (<1 kbp) cannot be attached. This precludes the future use of PCR-generated targets from ESTs, STSs etc. I have shown that fragments below 500 bp can be immobilized by washing the dried targets with poly-L-lysine. However, this chemistry produces a small, but unacceptable amount of nonspecific probe binding and I will pursue avenues to correct this. It is likely that the nonspecific binding is due to a small amount of free amines from the poly-L-lysine. Attempts will be made to block these groups, including using acetic anhydride or herring sperm/tRNA as blocking nucleic acids. If this is successful, attempts will be made to further stabilize the spots so that the reuse of the arrays may be possible. Bisulfite-catalysed transamination is an established method to link amino groups to the N-4 position of cytidine residues (13) and will be used to

form a covalent DNA/poly-L-lysine target 'gel'. Other approaches such as glutaraldehyde crosslinking will be attempted if necessary.

Method validation for printed arrays. I have demonstrated that the printer prototype shown in Figs # and # can print spots with reproducible hybridization properties from identical λ target solutions. It remains to be seen, however, how this printing process will perform using clones that differ in length, purity or viscosity. To address this issue, I have started to assemble the ~30 clones of the chromosome 20 scanning array into an 864 well plate. BT474 will be used as a test genome and results compared to our previous data (Fig 2).

Automation of the printing process. If the above validation is successful, the printing head shown in Fig. 4 will be attached to a robot (Western Technologies) that we have purchased and is currently being tested at Lawrence Berkeley Laboratory. It is expected that a significant amount of my time will needed to be spent fine tuning the steps involved in printing dense arrays (100-10,000 clone) onto large numbers (~100) of slides.

Application to large numbers of tumor samples. To define the minimum region of amplification on chromosome 20q, arrays will be prepared that contain the ~30 clones of the low resolution 'chromosome 20 scanning array' as well as ~30 clones that form a contig at 20q13. ~100 SPORE breast tumor samples will be screened on this array, and correlations will be made between clinical status and CNAs if possible. Arrays for this effort will be prepared with the optimum chemistry manually, with the desktop prototype, and with the automated printer as these instruments come on line.

Conclusions

The ability to rapidly scan cancer genomes for copy number abnormalities (CNAs) with high resolution will permit the identification of genes that are important to the disease process. Under the support of the US Army Medical Research and Material Command, I have been developing methods to detect such abnormalities and have been applying these methods to defining CNAs on chromosome 20 in breast cancer towards defining the critical gene(s) involved. In this method, tumor and normal DNA are labeled with different chromophores and simultaneously hybridized to an array of DNA clones spanning the region of interest. Copy number changes in the tumor genome are inferred by differences in the fluorescence ratio at a given clone.

My original proposal presented a research plan to develop technical aspects of this process, including the clone attachment chemistry, method validation in model systems and application to tumor genomes. Significant advances have been made in the stability of attached clone attachment and the validation of our array hybridization protocols has been established. Several breast tumors has been scanned for CNAs along chromosome 20 and display at least five regions of amplification and possibly one deleted region.

The statistical interdependence and significance of these regions requires the analysis of large numbers of tumors with higher resolution in the near future. For these reasons, and to make the technology generally applicable to the whole human genome, I have been developing an automatable array printer that is expected to manufacture large numbers of arrays of high density within the coming year.

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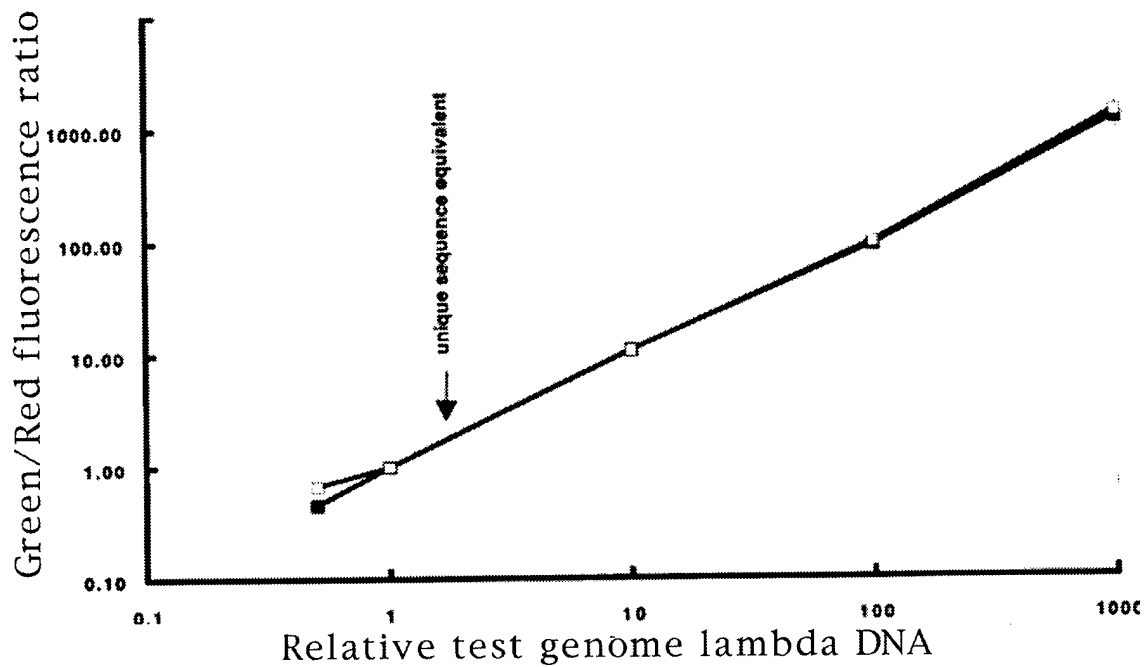


Figure 1. Probe titration experiment using λ as a test sequence. The fluorescence ratios are given for duplicate experiments where 0, 1, 2, 20 and 2000 pg of FITC-labeled λ DNA was hybridized against 20 pg of Texas Red-labeled λ DNA in a background of 200 ng of human genomic DNA. The arrow indicates probe concentration equivalent a single copy sequence (~ 2 pg) under these conditions.

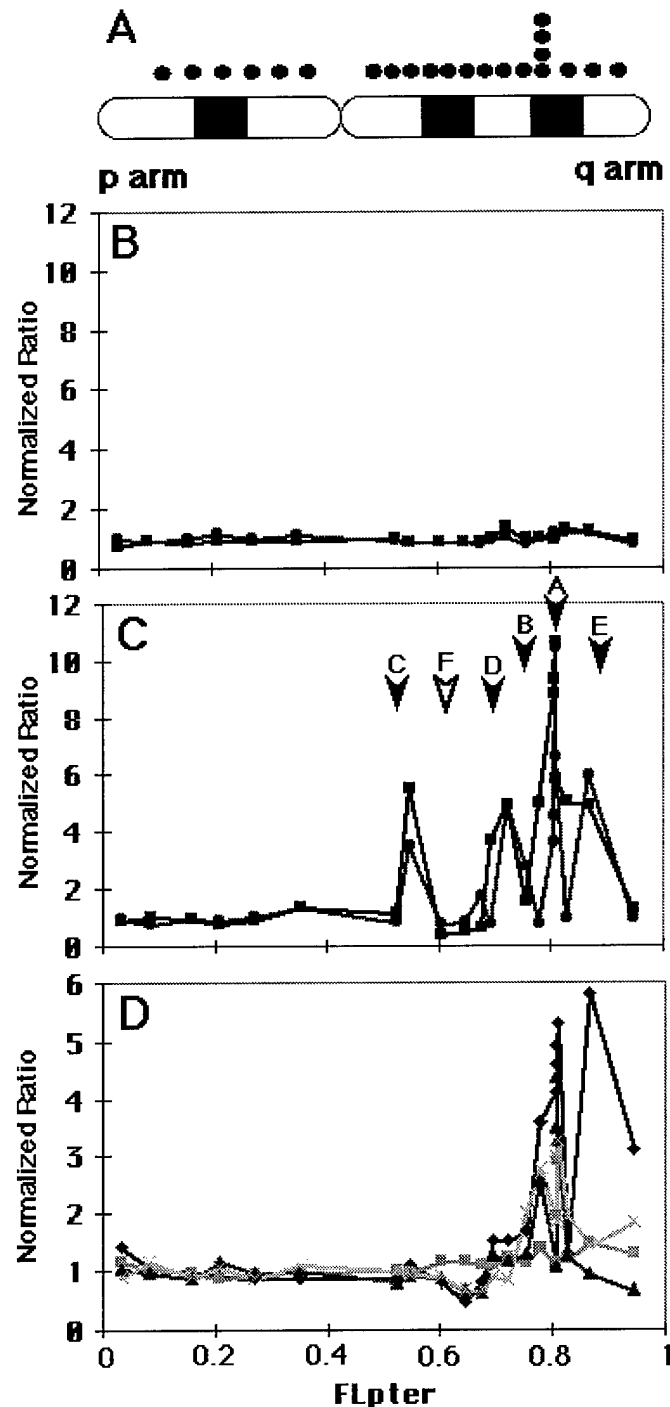


Figure 2. Array CGH results from five breast tumor and cell line BT474 using the chromosome 20 scanning array'. A. Clones in the array relative to the chromosome 20 ideogram. B. Replicate control hybridizations of normal male and normal female DNA. C. Breast tumor cell line BT474 (black) and a breast tumor S50 (red) indicate 5 regions of amplification (A-E) occurring in both samples and a region of copy number loss (F) occurring in BT474. D. Breast tumors S6 (dark blue), S21 (light blue), S59 (red) and S234 (green).

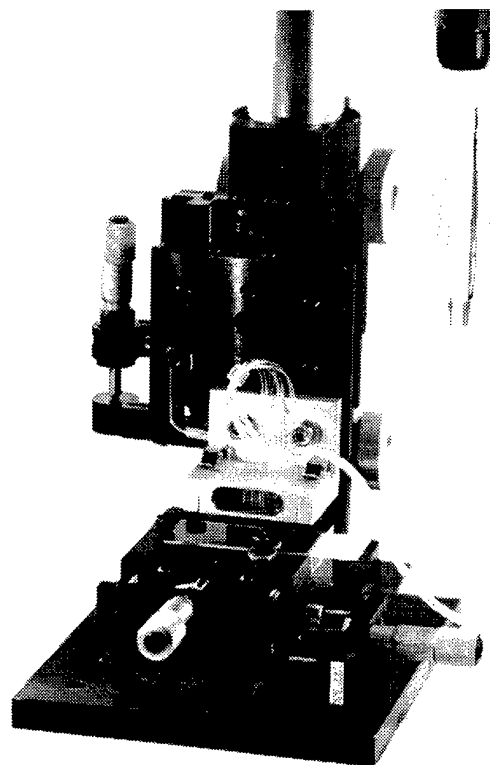


Figure 3. Prototype array printer.

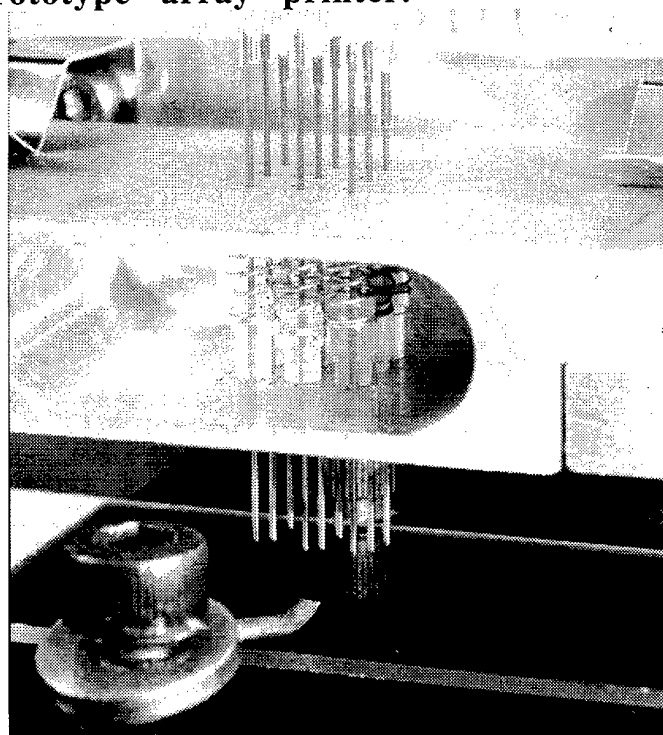


Figure 4. Closeup of the printing head. The 3 x 3 spring-loaded capillaries ($365\ \mu\text{m}$ o.d. x $75\ \mu\text{m}$ i.d. x 3 cm.) are spaced 3 mm apart in order to load from an 864-well plate.

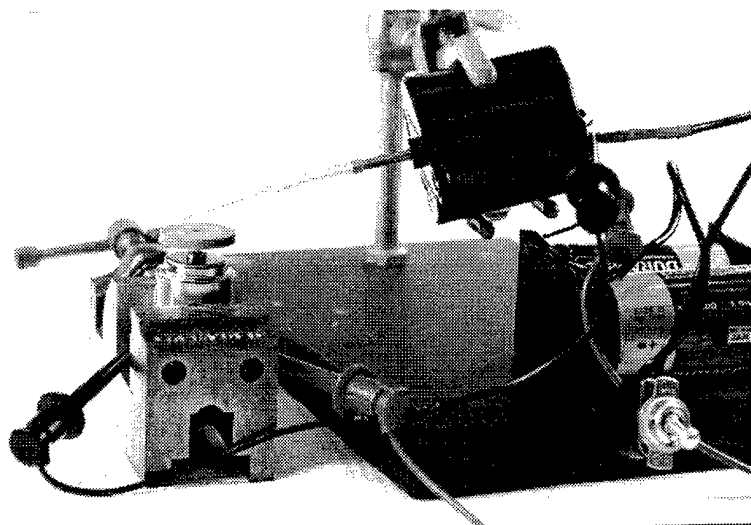


Figure 5. The lathe used to polish the array printing tips. The capillary is connected by a flexible tube to the 120 rpm motor in the upper right and polished by the quickly rotating ($\sim 5,000$ rpm) wheel shown at the left.

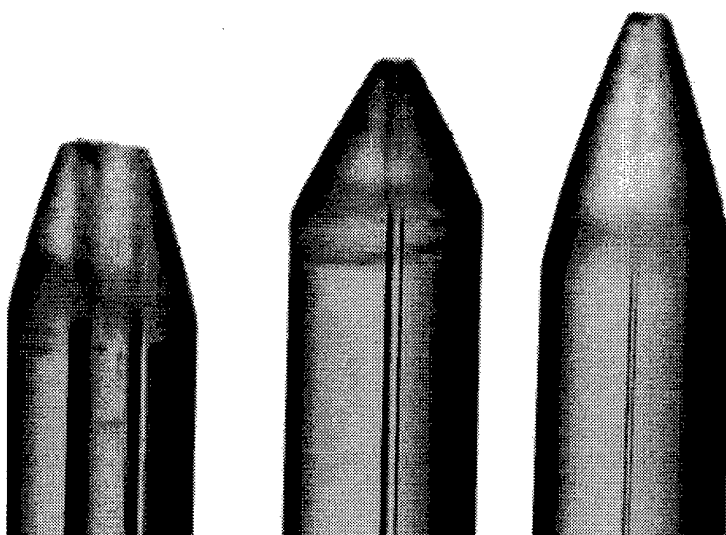


Figure 6. Closeup of the printing tips. The capillaries were polished using a homemade minirature lathe and posses i.d.s of 100, 25 and 10 μm and an o.d. of 365 μm

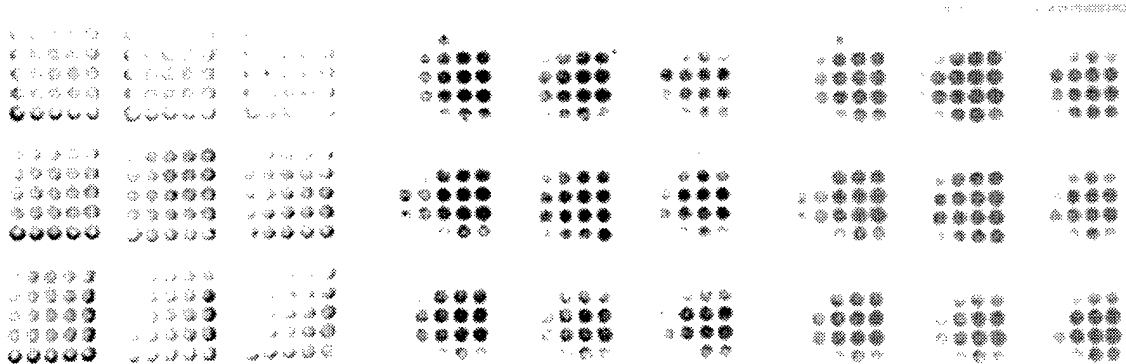


Figure 7. 225-spot test λ array with 500 μm spacing. From left to right are the DAPI, FITC and Texas Red images. This array was printed with 100 μm i.d. capillaries. The flat field problems seen here in the FITC and Texas Red channels have subsequently been corrected such that the variation in the Green/Red ratio across the image are is <10%.

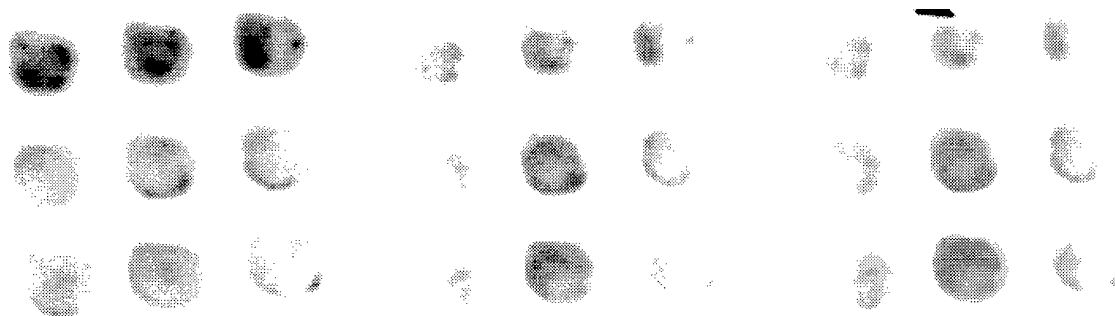


Figure 8. λ spots with 100 μm spacing. From left to right are the DAPI, FITC and Texas Red images. This array was printed with 25 μm i.d. capillaries.

Publications

Comparative Genomic Hybridization to DNA Microarrays: High Resolution Analysis of Complex Patterns of Deletion and Amplification in Breast Cancer, D. Pinkel, R. Seagraves, D. Sudar, S. Clark, I. Poole, D. Kowbel, C. Collins, W.-L. Kuo, C. Chen, Y. Zhai, S. H. Dairkee, B-M., Lejung, J. W. Gray, D. G. Albertson. *Manuscript in preparation.*

Meeting Abstracts

"An Era of Hope" Breast Cancer meeting. 31 Oct-3 Nov, 1997.
Washington D.C.

Amplification of the 20q region occurs in 15-20% of primary breast tumors and amplification of this region correlates with poor prognosis. Thus, we believe that 20q may harbor one or more genes involved in breast tumor development. We have developed comparative genomic hybridization to genomic clone arrays to quantify the copy number and location of amplified regions with high precision. Tumor and reference DNAs are labeled with different fluorochromes and simultaneously hybridized to an immobilized set of 30 mapped cosmids, BACs and PACs spanning the entire length of chromosome 20. Arrays are produced by capillary dispensing DNA/nitrocellulose/DMSO drops onto aminosilyated glass slides, forming ~200 micron spots. Copy number abnormalities involving a clone are indicated by the increase or decrease in the tumor/reference fluorescence ratio compared to average. By titrating lambda DNA probe in a background of human genomic DNA, we show that this method possesses sufficient sensitivity to detect single copy deletions as well as amplification, and is linear over several orders of magnitude. Analysis of breast cancer cell lines and primary tumors reveals at least four regions of amplification on 20q.

Personnel

The P.I. (Dr. Steven M. Clark) is the only person receiving pay from this effort